Glycolipids and Benzylammonium Lipids as Novel Antisepsis Agents: Synthesis and Biological Characterization

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New glycolipids and a benzylammonium lipid were rationally designed by varying the chemical structure of a D-glucose-derived hit compound active as lipid A antagonist. We report the synthesis of these compounds, their in vitro activity as lipid A antagonists on HEK cells, and the capacity to inhibit LPS-induced septic shock in vivo. The lack of toxicity and the good in vivo activity suggest the use of some compounds of the panel as hits for antisepsis drug development.

Mammalian cells have developed the ability to respond to minute (pM) quantities of endotoxin, unique, abundant, and generally conserved surface glycolipids (the entire LPS[a] and parts of its structure, lipid A) of Gram-negative bacteria.[1,2] This exceptional sensitivity is due to signal amplification mediated by a cascade of sequential protein−endotoxin and protein−protein interactions involving at least four extracellular and cell-surface host proteins: LPS-binding protein (LBP), soluble (s) and membrane-associated CD14, secreted and Toll-like receptor (TLR) 4-associated forms of MD-2, and TLR4 itself.[3−5] MD-2 plays an essential role in regulation of endotoxin mediated TLR4 activation, bridging recognition, normally initiated by CD14, to receptor activation or antagonism.[6−8] TLR4 trigger can be remarkably sensitive and robust, stimulating prompt and powerful host defense responses to different species of invading bacteria. However, an excessively potent host response generates life-threatening syndromes such as acute sepsis and septic shock. Gram-negative sepsis is the first cause of deaths in intensive care units, and it is associated to a mortality of about 45%.[9] Several pharmacologically active compounds have been developed and used in clinic to treat septic shock.[10] However, because of the serious side effects observed, there is still a major unmet medical need for safer and more effective antisepsis agents. The majority of small molecules so far proposed as leads for drug development are synthetic mimics of the lipid A or naturally occurring underacylated forms of lipid A that act as antagonists on the TLR4.[11] Among these compounds, worth noting is compound E5564,[12] a synthetic lipid A analogue that is currently in phase III clinical trials. The ethyl(6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242)[13] is another small molecule that reached the clinical phase III as antisepsis agent, and it was discovered by screening a large library of compounds. The antiendotoxic activity of this compound has been related to selective inhibition of the intracellular signaling of the TLR4 pathway. Another class of antisepsis agents directly target LPS by binding and sequestering it. LPS can be complexated and neutralized by antibodies,[14] nonantibody proteins,[15] and by small molecules such as the antibiotic cyclic peptide polymixin B[16] (PMB), a highly active endotoxin-sequestring agents that has never been used in a clinic because its high toxicity. Synthetic polycationic amphiphiles were shown to protect animals against endotoxin-induced lethality, and it was found that binding to LPS is a necessary but not sufficient condition for the inhibition of endotoxic activity.[17] Among these compounds are notable linear or branched[18] spermine derivatives and "Gemini" amphiphiles;[19] this last class of molecules has been designed as surfactant and therefore presents high cytotoxicity and hemolytic activity.

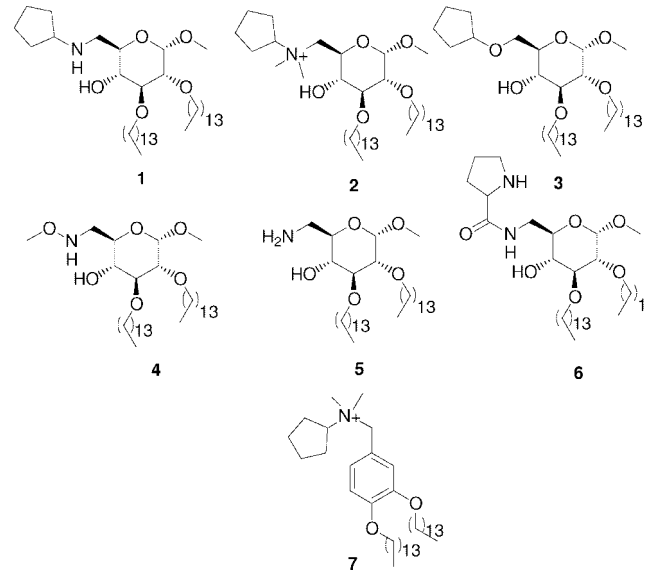

Figure 1. Structure of Glycolipids **1**−**6** and of benzylammonium lipid **7**.

We recently published the synthesis and the biological activity of compounds **1** and **2**, derived from the natural monosaccharide D-glucose[20] (Figure 1). These glycolipids have some structural elements in common with the branched spermine derivatives cited above,[18] namely two lipophilic (C_{14}) appendages linked to a positively charged amino group. We reported the activity of these compounds in inhibiting the lipid A-stimulated cytokine production in cells of the innate immune system such as macrophages and dendritic cells. Conversely, TNF-α production inhibition was not observed in macrophages and DC when these cells were treated with agents that selectively stimulate other TLRs. Compound **2**

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[a] Abbreviations: AcOEt, ethyl acetate; ANOVA, analysis of variance; sAP, secreted alkaline phosphatase; CD14, cluster differentiation antigen 14; CIP, calf intestinal alkaline phosphatase; DC, dendritic cell; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMAP, *N*,*N*-dimethylaminopyridine; Fmoc, fluorenylmethoxycarbonyl; HEK, human embrionic kidney; HOBt, *N*-hydroxybenzotriazole; LBP, lipid binding protein; LPS, lipopolysaccharide; MD-2, protein MD-2; NF-kB, nuclear factor-kB; SEM, standard error media; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

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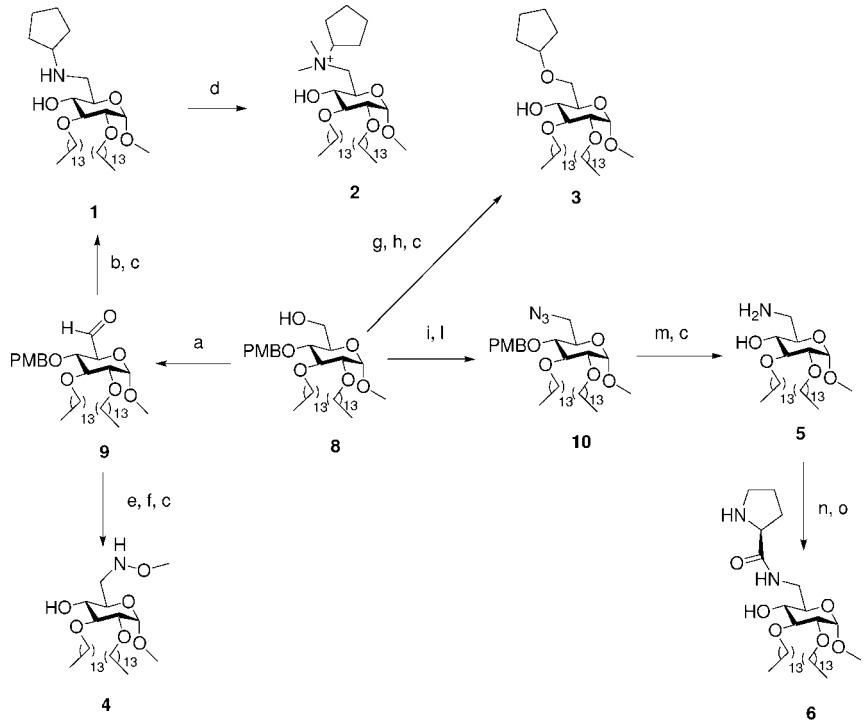
Scheme 1. Synthetic Route to Glycolipids **1**−**6**[a]

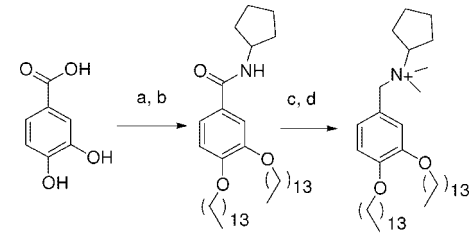


[a] Reagents and conditions: (a) Dess−Martin periodinane, CH_2Cl_2, rt, 1 h; (b) cyclopentylamine, $NaBH_3CN$, AcOH, CH_2Cl_2, MeOH, 24 °C, 12 h; (c) TFA, CH_2Cl_2, 24 °C, 1 h; (d) CH_3I, Na_2CO_3, DMF, 24 °C, 12 h; (e) $CH_3ONH_2 \cdot HCl$, pyridine, 24 °C, 12 h; (f) $NaBH_3CN$, AcOH, CH_2Cl_2, 24 °C, 2 h; (g) MsCl, pyridine, 24 °C, 5 h; (h) cyclopentanol, NaH, DMF, 24 °C, 12 h; (i) TsCl, DMAP, pyridine, 24 °C, 12 h; (l) NaN_3, tetrabutylamonium iodide (TBAI), DMF, 75 °C, 4 h; (m) PPh_3, H_2O, THF, 70 °C; (n) *N*-Fmoc-Pro, HOBt, DIC, DIPEA, DMF, 24 °C, 4 h; (o) piperidine/DMF 2:8, 24 °C, 1 h.

inhibited the lipid A-induced NF-kB activation of HEK cells selectively transfected with the human TLR4 while inactive on HEK cells transfected with human TLR9 gene. Taken together, these results suggest of a selective action of compound **2** on the TLR4 signal pathway. Recent evidence indicates a pivotal role of TLR4 in the development and maintenance of some other pathologies such inflammation[21] or painful neuropathies.[22] We observed an interesting activity of compound **2** in inhibiting both neuropathic pain[23] and carrageenan-induced acute local inflammation in animal models.[24]

To gain information about the structure−activity relationship for this new class of biologically active molecules, we designed compounds **3**−**7** that share certain common key features with compounds **1** and **2**. Chemical modifications of the lead **2** were focused on the amino group on C-6 of glucose and on its substituents. We designed compound **3**, in which the nitrogen atom on glucose C-6 has been replaced by the oxygen of a cyclopentyl ether, and compound **5**, conserving a primary amino group and lacking the cyclopentyl ring. In compound **4**, we inserted an *O*-methyl hydroxylamine function in C-6, while in compound **6**, the C-6 amine is condensed with a proline residue. The unique structure of this amino acid provides a secondary amine inserted into a five-membered cycle. In compound **7**, the cyclic core of the glucopyranose scaffold of **2** has been replaced by an aromatic ring. The mutual disposition of the C-6 nitrogen (or oxygen, in the case of **3**) and of the two tetradecyl ethers was conserved in all derivatives. The C-6, C-2, and C-3 positions of glucose of compounds **1**−**6** correspond to C-1, C-3, and C-4 positions of the benzene ring in **7**. The protonation state of the nitrogen could greatly influence the biological activity and the pharmacokinetic of these molecules, so all compounds but **3** are fully protonated and therefore positively charged at neutral pH. Compounds **1**−**7** were prepared starting from commercially available and inexpensive compounds: the methyl-β-D-glucopyranoside was transformed into glycolipids **1**−**6** according to the synthetic way shown in Scheme 1, whereas 3,4-dihydroxy benzoic acid was transformed in compound **7** through the four-step synthesis depicted in the Scheme 2.

Scheme 2. Four-Step Synthesis of Compound **7**[a]



[a] Reagents and conditions: (a) cyclopentylamine, HOBt, DIC, DIPEA, DMF, 24 °C, 12 h; (b) tetradecyl bromide, NaH, DMF, 60 °C, 2 h; (c) $LiAlH_4$, tetrahydrofuran (THF), 60 °C, 12 h; (d) CH_3I, Na_2CO_3, DMF, 24 °C, 3 h.

Methyl glucopyranoside **8**, protected at C-4 as *p*-methoxybenzyl (PMB) ether, and with two tetradecyl ether groups in C-2 and C-3, was prepared in gram quantities according to published procedures.[20] The oxidation of **8** to aldehyde **9** was carried out by treating with Dess−Martin periodinane. The reductive amination of **9** with cyclopentylamine and $NaBH_3CN$ followed by acidic cleavage of the PMB group afforded compound **1**.[20] The methylation of secondary amine group of **1** with iodomethane gave compound **2**[20] that directly precipitated in a pure form from the reaction crude after addition of light petroleum. Alternatively, the aldehyde group of **8** was converted to the corresponding *O*-methyl oxime derivative by reacting with $MeONH_2 \cdot HCl$ in pyridine. The *O*-methyl oxime was then reduced to hydroxylamine by treating with $NaBH_3CN$ and, after

removal of the PMB group with TFA, gave final compound **4**.[25] The C-6 hydroxyl of compound **8** was transformed into a mesylate by reacting with mesyl chloride in pyridine, then treatment with cyclopentanol in the presence of sodium hydride afforded compound **3** with a cyclopentyl ether in C-6. The reaction of **8** with *p*-toluensulfonyl(tosyl) chloride afforded the C-6 tosylate that was converted into **10** by nucleophilic displacement by using sodium azide. The conversion of the C-6 azide into a primary amine was accomplished in a clean and efficient way by treating **10** with triphenylphosphine: the hydrolysis of the aza-yilide intermediate in THF−water afforded the corresponding amine. After cleavage of the PMB ether in C-4, compound **5** was obtained, which was condensed with *N*-Fmoc-L-proline in the presence of HOBt, DIC and the Hunig's base DIPEA. Finally, the cleavage of the Fmoc carbamate (20% piperidine in DMF) from proline nitrogen afforded compound **6**. This molecule showed an unexpected chemical instability when dissolved in organic solvents at 24 °C, and decomposition was observed in NMR samples. A half-life of about 48 h was calculated based on the disappearance of compound **6** proton signals in NMR spectra.

The activity of compounds **1**−**7** as inhibitors of the TLR4 signal pathway was tested in vitro using a HEK-Blue LPS Detection Kit (InvivoGen). HEK-Blue-4 cells are HEK293 cells stably transfected with TLR4, MD2, and CD14 genes. In addition, these cells stably express an optimized alkaline phosphatase gene engineered to be secreted (sAP), placed under the control of a promoter inducible by several transcription factors such as NF-kB and AP-1. This reporter gene allows monitoring of the activation of TLR4 signal pathway by endotoxin (LPS or lipid A). Moreover, using HEK-Blue detection medium, phosphatase activity can be quantified spectrophotometrically. Therefore, we exploited this simple, fast, and reliable system to screen our compounds for their TLR4 antagonistic activity. HEK-Blue-4 cells were stimulated with lipid A (10 nM) after pre-exposure to the molecules, at three different concentrations (1, 5, and 10 μM) and phosphatase activity was measured by spectrophotometric analysis (630 nm, 24 h later). As shown in Figure 2, compounds **3** and **4** did not interfere with lipid A activity at least at the maximum concentration employed and compound **1** showed a weak inhibition (23%) only at the highest concentration (10 μM). On the other hand, compounds **2**, **5**, **6**, and **7** exhibited a significant inhibition of the lipid A activity decreasing the phosphatase activity in a concentration-dependent manner. It was possible to calculate a linear regression relating the inhibitory activity and the compound concentration (for compound **2** $r^2 = 0.5624$ $p < 0.05$; for compound **5** $r^2 = 0.6810$, $p < 0.01$; for compound **6** $r^2 = 0.6389$, $p < 0.05$; for compound **7** $r^2 = 0.8553$, $p < 0.001$). The IC_{50} comparison (Table 1) demonstrates that in this experiment **7** is the most active compound in suppressing lipid A activity: its potency is more than 3-fold greater than that of compounds **2**, **5**, and **6**.

It was important to verify that the observed inhibition in sAP activity is not a consequence of direct interference of compounds **1**−**7** with the enzyme. Control experiments were done using a similar enzyme, the recombinant calf intestinal alkaline phosphatase (CIP), by incubating molecules **2**, **5**, **6**, and **7** (10 μM) with different concentrations of CIP (0.4, 0.2, 0.02 mU/mL) in the presence of HEK-Blue detection medium. Recombinant CIP resulted active even at 0.02 mU/mL, demonstrating that compounds tested were not able to inhibit phosphatase activity (data not shown). An additional experiment was done to asses if compounds **2**, **5**, and **7** interfere with sAP produced by HEK-Blue. Molecules were incubated with supernatant containing HEK-Blue sAP produced with 24 h cells stimulation with lipid A (10 nM) and no enzyme inhibition could be detected (Supporting Information).

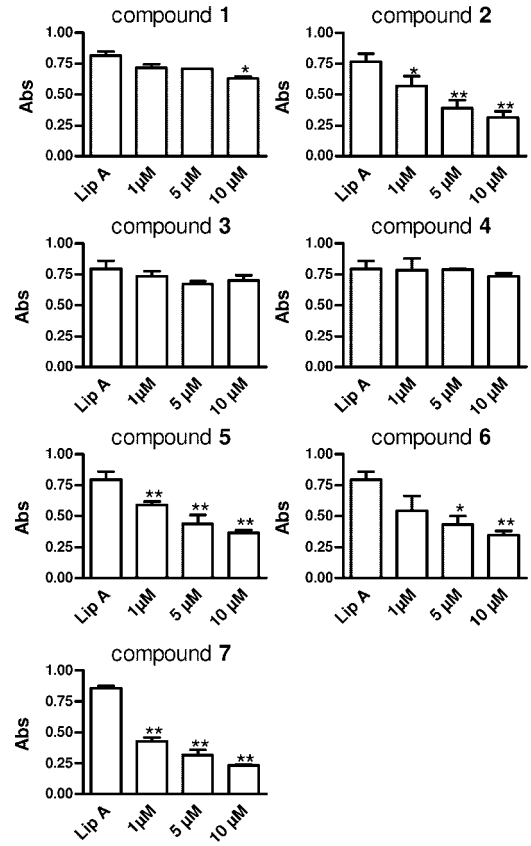


Figure 2. In vitro TLR4 antagonistic activity of compounds **1**−**7**. HEK-Blue-4 cells stably transfected with TLR4 and an optimized alkaline phosphatase gene engineered to be secreted (sAP) were treated with lipid A (10 nM) alone or lipid A in the presence of one among compounds **1**−**7** at three different concentrations (1, 5, 10 μM). NF-kB activation was evaluated by measuring phosphatase activity, expressed as absorbance (Abs) at 630 nm. The results are representative for three independent experiments and data are expressed as mean ± SEM *$p < 0.05$; **$p < 0.01$ (ANOVA; Dunnett's test).

Table 1. IC_{50} on Lipid A-Induced TLR4 Activation

compd	IC_{50} (μM)	confidence intervals
1	>10	
2	5.013	3.430−7.327
3	>10	
4	>10	
5	5.487	3.102−9.705
6	5.871	3.531−9.726
7	1.675	0.910−3.084

The cytotoxic potential of compounds **2**, **5**, **6**, and **7** was investigated. HEK-Blue-4 cells were grown in the presence of molecules at highest concentration (10 μM). After 24 h growth, the viability was evaluated using CellTiter Blue assay (Promega). As shown in Figure 3, for the HEK-Blue-4 cell line, no inhibitory effect of compounds **2**, **5**, and **6** on cell viability was detected. Compound **7** showed a diminution of the cell viability of about 17%; however, there is no statistical difference between the percentage of vitality cell in presence and absence of compound **7**, as demonstrated by Kruskal−Wallis ANOVA for non parametric data.

Finally, we tested active compounds **1**, **2**, **5**, and **7** for their capacity to protect animal against LPS-induced lethality. For the lethal endotoxin shock model, C57BL/6J male mice were

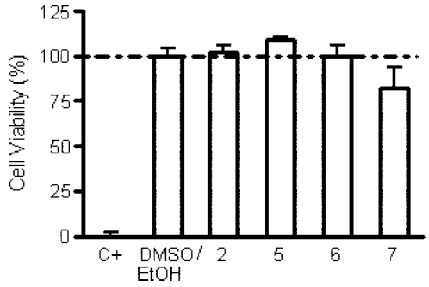


Figure 3. Viability of HEK-Blue-4 cells in the presence of active compounds **2**, **5**, **6**, and **7**. Cells were seeded and grown for 24 h in the presence of the most effective molecules at maximum concentration (10 μM). The cell viability was determined using CellTiter Blue cell viability assay (Promega). As controls, we set up triplicate wells with cells and 0.5% DMSO/EtOH and triplicate wells containing cells treated with a compound known to be toxic to the cells (C+). Kruskal−Wallis ANOVA for nonparametric data has been applied to analyze the differences among groups.

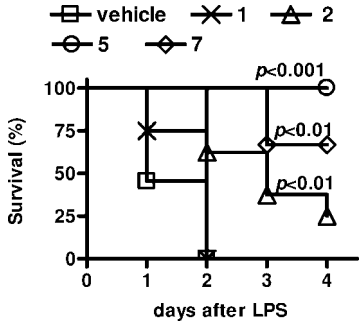


Figure 4. Effect of the different compounds on LPS-induced lethality in mice. Compounds were administered ip at 10 mg/kg, 30 min before LPS challenge. Statistical analysis was performed using the log rank test for nonparametric data. Each group consisted of eight mice.

intraperitoneally (ip) injected with 20 mg/kg LPS (from *E. coli* 055:B5, Sigma, Italy), and survival of mice was observed over 4 days. Compounds were administered ip 30 min before the LPS. As shown in Figure 4, ip injection of 20 mg/kg LPS was lethal to mice and all mice died within two days.

The administration of 10 mg/kg of the three molecules significantly increased survival of mice. Particularly, the compound **2** increased the survival rate from 0% to 25%, compound **7** from 0% to 67%, and all mice that received compound **5** survived. We further studied the protection from LPS induced lethality exerted by compound **5**, and we found that also a smaller dose (3 mg/kg) evoked a 100% survival of mice treated with LPS (data not shown). Further work is in progress in order to establish the smallest effective dose of compound **5** and to test whether the compound rescues mice even when administered after the LPS challenge. Compound **1**, which has the weakest TLR4 antagonistic activity in vitro, was not effective either in the lethal endotoxin shock model. All mice treated with this compound died within the second day, exactly as mice treated with LPS alone.

Compounds **2**, **5**, and **7** were tested to assess their specificity for TLR4 signal pathway. HEK-Blue-4 cells were grown with TNF-α as a stimulus for TLR4 independent production of sAP in presence of compounds. Results are depicted in Figure 5 and clearly show no inhibitory effects on TNF-α induced activation.

By comparing the in vitro and in vivo activity of compounds **1**−**7**, it is possible to elucidate some aspects of structure−activity relationship in this series. Compounds **3** and **4** lacking a protonatable amino group were inactive in antagonizing the lipid A effect on HEK cells. Compound **6** is relatively unstable when dissolved in solution at rt:

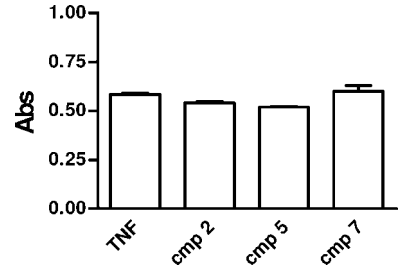


Figure 5. In vitro TNFα activation of HEK-Blue-4 cells. HEK-Blue-4 cells were treated with TNF-α (1 ng/mL) alone or in the presence of compounds **2**, **5**, and **7** (10 μM). sAP production was evaluated by measuring absorbance (Abs) at 630 nm.

its half-life of about 48 h is compatible with the in vitro tests duration but significant degradation is observed after the time required for in vivo tests (5 days). We are investigating the mechanism of degradation of this compound and, once clarified, we will be able to design chemically stable structural variants. Glycolipids **2**, **5**, and **6** are active lipid A antagonists on HEK cells and protect mice from LPS-induced lethality in a statistically significant way. Notably, compound **5** is the most active in vivo and the 100% survival observed at 3 mg/kg dose suggests a possible use of this compound at lower concentrations. The potency of **5** is comparable to that of the best antisepsis agents so far developed (compound TAK-242, in clinical phase III, showed comparable activity in this test with an ED_{50} of 0.3 mg/kg).[13] The second most potent compound in vivo and the most potent in vitro is molecule **7**, with an aromatic ring instead of the pyranose ring of glucose. Both compounds **5** and **7** have been selected for clinical experimentation on the base of their in vivo activity and lack of toxicity on cells. We are currently investigating the mechanism of action of these compounds, and we are trying to identify their specific target(s) in the TLR4 signaling pathway. Sepsis is a very complex disease involving various cascades of events and many factors. For this reason, further work is necessary to prove the potential clinical significance of our compounds, especially their efficacy to improve survival even when administered after the onset of the pathology.

Experimental Section

Methyl 6-Deoxy-6-amino-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (5). To a solution of methyl 6-deoxy- 6-amino-4-*O*-(4′-methoxybenzyl)-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (0.25 g, 0.35 mmol) in CH_2Cl_2 (5 mL), TFA (3.3 mL) was added and the solution was stirred at rt for 2 h. Solvents were evaporated in vacuo, the residue was purified by flash chromatography on silica gel (gradient starting from AcOEt/MeOH/water 8: 2: 0.1), and pure product was obtained as a colorless oil (0.18 g, 90% yield). Anal. ($C_{35}H_{71}NO_5$) C, H, N.

Methyl 6-Deoxy-6-[(2-pyrrolidinyl)carbonylamino]-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (6). A solution of methyl 6-deoxy-6-[[(1-fluorenylmethoxycarbonyl)-2-pyrrolidinyl]carbonylamino]-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (0.09 g, 0.100 mmol) in piperidine/DMF 2:8 (4 mL) was stirred at rt for 1 h. After this time, reaction was complete, as assessed by TLC analysis (AcOEt, Rf product = 0.12). Solvent was evaporated in vacuo, the residue was purified by flash chromatography on silica gel (gradient starting from petroleum AcOEt/MeOH 8:2), and pure product was obtained as a colorless oil (0.06 g, 90% yield). Anal. ($C_{40}H_{78}N_2O_6$) C, H, N.

***N*-(3,4-Bis-tetradecyloxy-benzyl)-*N*-cyclopentyl-*N*,*N*-dimethyl-ammonium (7).** To a solution of *N*-(3,4-bis-tetradecyloxy-benzyl)-*N*-cyclopentylamine (0.3 g, 0.5 mmol) in anhydrous DMF (50 mL), iodomethane (70 μL, 1 mmol) and Na_2CO_3 (160 mg, 1.5 mmol) were added and the solution was heated at 60 °C for 1 h. After this time, reaction was complete, as assessed by TLC analysis ($CHCl_3$/MeOH 9:1, R_f product = 0.47). Solvent was evaporated in vacuo, the residue was dissolved in chloroform (40 mL) and washed with brine (2 × 40 mL). The organic layer was dried on sodium sulfate, filtered, and the

solvent evaporated. Pure product was recovered by trituration in cold light petroleum (300 mg, 95% yield). Anal. ($C_{40}H_{78}NO_2$) C, H, N.

In Vitro Experiments. All compounds were dissolved in DMSO/ethanol 1:1 and then diluted in PBS and added 20 μL per well of a flat-bottom 96-well plate at three different concentrations (1, 5, 10 μM). The final organic solvent (DMSO/ethanol) concentration per well is less than 0.5%. HEK-Blue-4 cells were detached by the use of diluted trypsin-EDTA solution (1/3 in PBS), and the cell concentration was estimated by using a counting cell. The cells were diluted in HEK-Blue detection medium (InvivoGen), and 200 μL of cell suspension (20000 cells) were added to each well. One hour after incubation with compounds at 37 °C in a CO_2 incubator, cells were stimulated with 20 μL of lipid A (0.01 μM) or with TNFα (1 ng/mL) per well. The plate was incubated at 37 °C in a CO_2 incubator for 24 h. Lipid A and TNFα induce TLR4 pathway activation, leading to alkaline phosphatase secretion. The phosphatase activity is detected by the use of HEK-Blue detection medium, and it can be quantified spectrophotometrically: the plate reading was assessed by using a spectrophotometer set on 630 nm. As positive control, we treated the cells with lipid A (0.01 μM) or TNFα (1 ng/mL) alone.

Lethal Endotoxin Shock Model. For the lethal endotoxin shock model, C57BL/6J male mice (9 weeks old, Harlan Italy) were intraperitoneally (ip) injected with 20 mg/kg LPS (from *E. coli* 055: B5, Sigma, Italy), and survival of mice was observed over 4 days. All compounds were dissolved in ethanol:saline (1:9) and administered ip 30 min before the LPS.

Supporting Information Available: General experimental methods, synthetic procedures, complete spectroscopic characterization of target molecules (^{1}H, ^{13}C NMR, MS), full details for the experiments on HEK cells and mice. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Beutler, B.; Jiang, Z.; Georgel, P.; Crozat, K.; Croker, B.; Rutschmann, S.; Du, X.; Hoebe, K. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.* **2006**, *24*, 353–389.

(2) Ulevitch, R. J.; Tobias, P. S. Recognition of Gram-negative bacteria and endotoxin by the innate immune system. *Curr. Opin. Immunol.* **1999**, *11*, 19–22.

(3) Freudenberg, M. A.; Tchaptchet, S.; Keck, S.; Fejer, G.; Huber, M.; Schuetze, N.; Beutler, B.; Galanos, C. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. *Immunobiology* **2008**, *213*, 193–203.

(4) Gioannini, T. L.; Teghanemt, A.; Zhang, De, S.; Levis, E. N.; Weiss, J. P. Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation. *J. Endotoxin Res.* **2005**, *11*, 117–123.

(5) Jerala, R. Structural biology of the LPS recognition. *Int. J. Med. Microb.* **2007**, *297*, 353–363.

(6) Teghanemt, A.; Widstrom, R. L.; Gioannini, T. L.; Weiss, J. P. Isolation of Monomeric and Dimeric Secreted MD-2: endotoxin-sCD14 and toll-like receptor 4 ectodomain selectively react with the monomeric form of secreted MD-2. *J. Biol. Chem.* **2008**, *283*, 21881–21889.

(7) Teghanemt, A.; Prohinar, P.; Gioannini, T. L.; Weiss, J. P. Transfer of Monomeric Endotoxin from MD-2 to CD14: characterization and functional consequences. *J. Biol. Chem.* **2007**, *282*, 36250–36256.

(8) Gioannini, T. L.; Teghanemt, A.; Zhang, De, S.; Coussens, N. P.; Dockstader, W.; Ramaswamy, S.; Weiss, J. P. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4186–4191.

(9) Martin, G. S.; Mannino, D. M.; Eaton, S.; Moss, M. The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.* **2003**, *348*, 1546–1554.

(10) (a) Bernard, G. R.; Vincent, J. L.; Laterre, P. F.; LaRosa, S. P.; Dhainaut, J. F.; Lopez-Rodriguez, A.; Steingrub, J. S.; Garber, G. E.; Helterbrand, J. D.; Ely, E. W.; Fisher, C. J. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N. Engl. J. Med* **2001**, *344*, 699–709. (b) Warren, H. S.; Suffredini, A. F.; Eichacker, P. Q.; Munford, R. S. Risks and benefits on activated protein C treatment for severe sepsis. *N. Engl. J. Med* **2002**, *347*, 1027–1030.

(11) (a) Hawkins, L. D.; Christ, W. J.; Rossignol, D. P. Inhibition of endotoxin response by synthetic TLR4 antagonists. *Curr. Top. Med. Chem.* **2004**, *4*, 1147–1171. (b) Christ, W. J.; Hawkins, L. D.; Lewis, M. D.; Kishi, Y. Synthetic lipid A antagonists for sepsis treatment. *Carbohydr.-Based Drug Discovery* **2003**, *1*, 341–355. (c) Christ, W. J.; Asano, O.; Robidoux, A. L.; Perez, M.; Wang, Y.; Dubuc, G. R.; Gavin, W. E.; Hawkins, L. D.; McGuinness, P. D.; Mullarkey, M. A. E5531, a pure endotoxin antagonist of high potency. *Science* **1995**, *268*, 80–83. (d) Martin, O.; Zhou, W.; Wu, X.; Front-Deschamps, S.; Moutel, S.; Schindl, K.; Jeandet, P.; Zbaeren, C.; Bauer, J. A. Synthesis and Immunobiological Activity of an Original Series of Acyclic Lipid A Mimics Based on a Pseudodipeptide Backbone. *J. Med. Chem.* **2006**, *49*, 6000–6014.

(12) E5564: α-D-Glucopyranose-3-*O*-decyl-2-deoxy-6-*O*-[2-deoxy-3-*O*-[(3*R*)-3-methoxydecyl]-6-*O*-methyl-2-[[(11*Z*)-1-*oxo*-11-octadecenyl]amino]-4-*O*-phosphono-D-glucopyranosyl]-2-[(1,3-dioxotetradecyl)amino]-1-(dihydrogen phosphate), tetrasodium salt. Rossignol, D. P.; Lynn, M. Antagonism of in vivo and ex vivo response to endotoxin by E5564, a synthetic lipid A analogue. *J. Endotoxin Res.* **2002**, *8*, 483–488.

(13) (a) Yamada, M.; Ichikawa, T.; Ii, M.; Sunamoto, M.; Itoh, K.; Tamura, N.; Kitazaki, T. Discovery of novel and potent small-molecule inhibitors of NO and cytokine production as antisepsis agents: Synthesis and biological activity of alkyl 6-(*N*-substituted sulfamoyl)-cyclohex-1-ene-1-carboxylate. *J. Med. Chem.* **2005**, *48*, 7457–7467. (b) Ii, M.; Matsunaga, N.; Hazeki, K.; Nakamura, K.; Takashima, K.; Seya, T.; Hazeki, O.; Kitazaki, T.; Iizawa, Y. A novel cyclohexene derivative, ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. *Mol. Pharmacol.* **2006**, *69*, 1288–1295.

(14) Cross, A.; Opal, S. M. Therapeutic intervention in sepsis with antibody to endotoxin: is there a future? *J. Endotoxin Res.* **1994**, *1*, 57–59.

(15) (a) Leturcq, D. J.; Moriarty, A. M.; Talbott, G.; Winn, R. K.; Martin, T. R.; Ulevitch, R. J. Therapeutic strategies to block LPS interactions with its receptor. *Prog. Clin. Biol. Res.* **1995**, *392*, 473–477. (b) David, S. A.; Balaram, P.; Mathan, V. I. Characterization of the interaction of lipid A and lipopolysaccharide with human serum albumin: Implications for an endotoxin-carrier function for albumin. *J. Endotoxin Res.* **1995**, *2*, 99–106.

(16) (a) Storm, D. R.; Rosenthal, K. Polymyxin and related peptide antibiotics. *A Rev. Biochem.* **1977**, *46*, 723–763. (b) Morrison, D. C.; Jacobs, D. M. Binding of Polymyxin B to the lipid A portion of bacterial polysaccharides. *Immunochemistry* **1976**, *13*, 813–818.

(17) (a) David, S. A.; Silverstein, R.; Amura, C. R.; Kielian, T.; Morrison, D. C. Lipopolyamines: Novel antiendotoxin compounds that reduce mortality in experimental sepsis caused by Gram-negative bacteria. *Antimicrob. Agents Chemother.* **1999**, *43*, 912–919. (b) David, S. A. Towards a rational development of anti-endotoxin agents: novel approaches to sequestration of bacterial endotoxin with small molecules. *J. Mol. Recognit.* **2001**, *14*, 370–387. (c) Burns, M. R.; Jenkins, S. A.; Wood, S. J.; Miller, K.; David, S. A. Structure−activity relationships in lipopolysaccharide neutralizers: Design, synthesis, and biological evaluation of a 540-membered amphipathic bisamide library. *J. Comb. Chem.* **2006**, *8*, 32–43.

(18) (a) Miller, K. A.; Kumar, E. V. K.; Wood, S. J.; Cromer, J. R.; Datta, A.; David, S. A. Lipopolysaccharides sequestrants: structural correlates of activity and toxicity in novel acylhomospermines. *J. Med. Chem.* **2005**, *48*, 2589–2599. (b) Burns, M. R.; Jenkins, S. A.; Kimbrell, M. R.; Balakrishna, R.; Nguyen, T. B.; Abbo, B. G.; David, S. A. Polycationic sulfonamides for the sequestration of endotoxin. *J. Med. Chem.* **2007**, *50*, 877–888.

(19) David, S. A.; Perez, L.; Infante, M. R. Sequestration of bacterial lipopolysaccharide by bis-arginine gemini compounds. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 357–360.

(20) Peri, F.; Granucci, F.; Costa, B.; Zanoni, I.; Marinzi, C.; Nicotra, F. Amino and Hydroxylamino Monosaccharides Inhibit Lipid A Stimulated Activation of Human Dendritic Cells and Macrophages. *Angew. Chem., Int. Ed.* **2007**, *46*, 3308–3312.

(21) O'Neill, L. A. When signaling pathways collide: positive and negative regulation of Toll-like receptor signal transduction. *Immunity* **2008**, *29*, 12–20.

(22) Tanga, F. Y.; Raghavendra, V.; De Leo, J. A. Quantitative real-time RTPCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem. Int.* **2005**, *45*, 397–407.

(23) Bettoni, I.; Comelli, F.; Rossini, C.; Granucci, F.; Giagnoni, G.; Peri, F.; Costa, B. Glial TLR4 Receptor as New Target to Treat Neuropatic Pain: Efficacy of a New Receptor Antagonist in A Model Peripheral Nerve Injury in Mice. *Glia* **2008**, *56*, 1312–1319.

(24) Peri, F.; Nicotra, F. Lipid A antagonists with anti-septic shock, antiinflammatory, anti-ischemia and analgesic activity. Patent PCT/EP2007/002279, 2007.

(25) Peri, F.; Marinzi, C.; Barath, M.; Granucci, F.; Urbano, M.; Nicotra, F. Synthesis and biological evaluation of novel lipid A analogues. *Bioorg. Med. Chem.* **2006**, *14*, 190–199.

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